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COVALENT BINDING STUDIES ON THE ^{14}C-LABELED ANTITUMOUR COMPOUND 2,5-BIS(1-AZIRIDINYL)-1,4-BENZOQUINONE. INVOLVEMENT OF SEMIQUINONE RADICAL IN BINDING TO DNA, AND BINDING TO PROTEINS AND BACTERIAL MACROMOLECULES IN SITU

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SUMMARY

2,5-Bis(1-aziridinyl)-1,4-benzoquinone (BABQ) is a compound from which several antitumour drugs are derived, such as Trenimone, Carboquone and Diaziquone (AZQ). The mechanism of DNA binding of BABQ was studied using ^{14}C-labeled BABQ and is in agreement with reduction of the quinone moiety and protonation of the aziridine ring, followed by ring opening and alkylation. The one-electron reduced (semiquinone) form of BABQ alkylates DNA more efficiently than two-electron reduced or non reduced BABQ. Covalent binding to polynucleotides did not unambiguously reveal preference for binding to specific DNA bases. Attempts to elucidate further the molecular structure of DNA adducts by isolation of modified nucleosides from enzymatic digests of reacted DNA failed because of instability of the DNA adducts. The mechanism of covalent binding to protein (bovine serum albumin, BSA) appeared to be completely different from that of covalent binding to DNA. Binding of BABQ to BSA was not enhanced by reduction of the compound and was pH dependent in a way that is opposite to that of DNA alkylation. Glutathione inhibits binding of BABQ to BSA and forms adducts with BABQ in a similar pH dependence as the protein binding. The aziridine group therefore does not seem to be involved in the alkylation of BSA. Incubation of intact *E. coli* cells, which endogenously reduce BABQ, resulted in binding to both DNA and RNA, but also appreciable protein binding was observed.

Abbreviations: BABQ, 2,5-bis(1-aziridinyl)-1,4-benzoquinone; BSA, bovine serum albumin.

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INTRODUCTION

After the discovery of tumour growth inhibiting effects of sulfur- and nitrogen mustard compounds, alkylating agents containing aziridinyl groups have been developed. The combination of aziridinyl and a benzoquinone moiety by Domagk et al. [1] yielded 2,5-bis(1-aziridinyl)-1,4-benzoquinone (BABQ, Fig. 1), a compound with remarkable antineoplastic properties, but also dose-limiting toxicity [2]. Later, a series of BABQ derivatives was screened by Driscoll et al. [3] for activity against brain tumours. From this series, Diaziquone (AZQ) was one of the most promising compounds. This compound has been used in many clinical phase I and II trials and appears to be active against brain tumours in man [4,5] and several other tumours. The BABQ compounds are (bio)reductively activated drugs, a term introduced by Lin et al. [6], like other antitumour agents containing a quinone moiety, such as adriamycin and mitomycin C (for a review, see Powis [7]). Reduction of the benzoquinone moiety strongly enhances the alkylating activity of BABQ derivatives [8]. Bioreductively activated drugs are of potential use against solid tumours, which contain hypoxic parts due to poor vascularization; hypoxia is thought to favour the reduced state of a compound, by slowing reoxidation [9]. Moreover, due to the aziridine ring BABQ derivatives are acid-activated to alkylating species. This may increase tumour cell specificity, as in some tumour tissues, especially hypoxic tissues, the pH is lower than in normal tissues [10,11].

Previously, we investigated the effects of DNA damage by a series of 3,6-substituted BABQ derivatives in a bacterial differential DNA repair test and a bacteriophage DNA inactivation test [12]. Further, we reported on DNA cross-linking by these compounds [8]. The parent compound BABQ showed a high capacity to inactivate DNA as well as to form DNA cross-links. To study DNA alkylation by this compound in more details, we synthesized ^{14}C-labeled BABQ.

In the present study, the dependence of DNA binding upon pH and reduction is investigated. We demonstrate that the one-electron reduced semiquinone form is mainly responsible for DNA alkylation. Base-sequence preference is investigated by studying binding to several synthetic polynu-

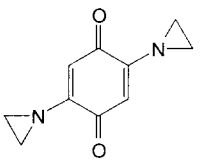

Fig. 1. Structure of 2,5-bis(1-aziridinyl)-1,4-benzoquinone (BABQ).

cleotides. Binding of BABQ to protein appears to proceed with a mechanism that is essentially different from that of DNA binding. To study covalent binding in intact cells, we measured bacterial DNA and protein binding in *E. coli* exposed to BABQ, which is activated by endogenous bacterial reductive enzymes.

MATERIALS AND METHODS

2,5-[^{14}C]Bis(1-aziridinyl)-1,4-benzoquinone was prepared from 2,3,5,6,-[^{14}C]-1,4-benzoquinone (17 mCi/mmol, from Amersham) as described in the literature [13] and diluted with unlabeled 2,5-bis(1-aziridinyl)-1,4-benzoquinone to obtain a specific activity of 5.6×10^9 Bq · mol^{-1}, determined by scintillation counting and spectrophotometry (the molar absorptivity at 324 nm was determined to be 1.75×10^4 M^{-1} · cm^{-1}). The radiochemical purity was checked by TLC analysis on Silica plates (Merck alufoil Kieselgel 60F254), developed in chloroform/methanol (10:1), followed by autoradiography. No other products were detectable on the autoradiogram. A stock solution containing 2.25 mM BABQ in dimethylformamide was prepared and stored at 4 °C in the dark. Calf thymus DNA was from Sigma Chemical Company (St. Louis, MO). Poly(dA) · poly(dT), poly(dA · dT), poly(dG) · poly(dC) and poly(dG · dC) were from Boehringer (Mannheim, F.R.G.). Identity and purity were confirmed by recording melting curves, [14]. Stock solutions of DNA and polynucleotides in water were 3.88 mM, calculated as phosphate and determined spectrophotometrically using extinction coefficients given by Wells et al. [14]. BSA, demineralized, was from Poviet Producten BV (Boxtel, The Netherlands). DNase I (EC 3.1.21.1.), nuclease P1 (EC 3.1.10.1) and alkaline phosphatase (EC 3.1.3.1.) were from Boehringer Mannheim GmbH. *E. coli* K12-343/753 ("wild type") and 343/765 (defective in *recA* and *uvrB* genes) were kindly provided by G.R. Mohn (R.I.V.M., Bilthoven, The Netherlands). For a full description of growing and maintenance of the strains, see Mohn et al. [15]. Ingredients for bacterial media were obtained from Difco Laboratories (Detroit, MI). Glucose-phosphate buffer contained 2.8 g of glucose per 100 ml in 60 mM phosphate buffer (pH 7.1). Doubly distilled water was used in all experiments. Methanol for HPLC was from Baker Chemicals. All other chemicals were analytical grade.

Scintillation counting was performed on a Packard Minaxi-β Tri-Carb 4430 Liquid Scintillation Counter, using Hydro-Luma scintillation cocktail (Lumac BV, Landgraaf, The Netherlands). Counting efficiency was determined by the internal channel ratio method. HPLC analyses were performed on a Spectra-Physics SP8400 HPLC system with a SP8440 UV-detector, using a Spherisorb S5-ODS2 column. UV-VIS spectra were recorded on a Perkin-Elmer Lambda-5 spectrophotometer. Fast atom bombardment (FAB) mass spectra were obtained with a VG-ZAB-2f mass spectrometer (VG-Analytical, Manchester, U.K.), equipped with a Saddlefield ion gun (1 mA, 7 kV) using xenon gas. Electrochemical reduction was performed under nitrogen bubbling in a vessel kept at 25 °C, using a Radiometer K401 saturated calomel electrode, a platinum wire auxiliary electrode, a 2-ml mercury pool as cathode and a

Brooker E100 as potentiostat, at a potential 200 mV more negative than the halfwave potential of the compound. Nitrogen was made oxygen-free photoreductively by passing the nitrogen through an illuminated solution containing 0.1 M pyrophosphate, 0.1 M EDTA, 4 mM methyl viologen and 40 μM proflavine, as described [16].

BABQ binding to DNA and polynucleotides

A mixture containing 225 μM [^{14}C]BABQ and 25 mM Tris-acetate buffer (pH 7.0) was electrochemically reduced. After reduction, 20 μl of this solution was added to a mixture of 20 μl DNA or polynucleotide stock solution and 4 μl Tris-acetate buffer of varying pH (1 M). After 90-min incubation at 37 °C, DNA and DNA-adducts were separated from BABQ and its decomposition products by Sephadex G-50 spun column filtration [17]. The Sephadex eluate was mixed with scintillation cocktail and counted. Control experiments without BABQ reduction have also been performed.

Time-dependence of BABQ binding to calf thymus DNA

A mixture containing 225 μM [^{14}C]BABQ, 0.6 mg · ml^{-1} calf thymus DNA (2.0 mM as phosphate) and 5 mM Tris-acetate buffer (pH 5.0) was electrochemically reduced under nitrogen at 25 °C. During reduction, the cathode reduction current was registrated. At timed intervals, samples of 100 μl were taken, filtrated over Sephadex G-50 columns by spun column filtration and assayed for amount of radiolabel. After 105 min, reduction was stopped, Tris-acetate buffer (pH 7.0) was added to a final concentration of 100 μM and the mixture was incubated under air at three temperatures: 25 °C, 37 °C and 45 °C. At timed intervals, samples of 100 μl were taken and treated as described above. Similar experiments were also performed with unreduced BABQ.

To study binding exclusively by fully (two-electron) reduced BABQ, a mixture containing 450 μM [^{14}C]BABQ and 5 mM Tris-acetate buffer (pH 7.0) was electrochemically reduced under nitrogen at 25 °C. After completion of reduction, an equal volume of a nitrogen-saturated solution containing 1.2 mg · ml^{-1} calf thymus DNA (3.9 mM as phosphate) in 200 mM Tris-acetate (pH 5.0) was added. Reduction was continued and at timed intervals, samples of 100 μl were taken and treated as described earlier in this section.

Analysis of BABQ-DNA adducts

BABQ-DNA adducts were obtained by electrochemical reduction under nitrogen, of a mixture containing 0.5 mg · ml^{-1} calf thymus DNA and 2 mM BABQ in 50 mM phosphate buffer (pH 6.0) for 60 min at 25 °C and subsequent reoxidation under air for 120 min at 25 °C. DNA containing BABQ adduct was separated from BABQ products by chromatography on a 20-ml Sephadex G-25 column and subsequent extractions with 1-butanol and diethyl ether. The yield of DNA adducts as judged from UV absorbance was approximately one adduct per 30 basepairs. DNA was enzymatically digested by incubating the solution with 100 units · ml^{-1} DNAse I and 10 μg · ml^{-1}

nuclease P1 in 50 mM acetate buffer (pH 5.5) containing 0.6 mM $ZnSO_4$ and 10 mM $MgSO_4$ for 3 h at 37 °C [18]. DNA degradation was followed by measuring the increase of absorbance at 260 nm (A_{260}). After 3-h incubation, the amount of DNase I and nuclease P1 was doubled and the digestion was continued for another 60 min. When no more increase in A_{260} was observed, the pH of the solution was raised to 8.0 by addition of a 1-M tris-solution. Alkaline phosphatase was added to a concentration of 2 units · ml^{-1} and the solution was incubated at 37 °C for 30 min. Undigested DNA was precipitated with ethanol and removed by centrifugation. The supernatant was concentrated in a Speedvac and submitted to HPLC analysis. Gradient elution was 8 performed from 100% buffer (100 mM acetate, pH 3.3) to 100% methanol in 60 min, flow rate 1 ml · min^{-1}. Detection was at 343 nm, the UV maximum of the DNA-BABQ adduct and 275 nm. Main adduct peaks were quantitatively collected by repeated injections, lyophilized and after dissolution again submitted to HPLC analysis, this time with gradient elution from 100% buffer (10 mM ammonium acetate, pH 7.2) to 100% methanol in 60 min, flow rate 1 ml · min^{-1}. UV spectra of the main adduct peaks were recorded. Products were again lyophilized and submitted to mass spectral analysis.

Binding of BABQ to albumin

A mixture containing 225 μM [^{14}C]BABQ and 10 mM Tris-acetate buffer (pH 7.0) was electrochemically reduced. After reduction 100 μl of this solution was added to a mixture of 100 μl of bovine albumin stock solution (15 mg · ml^{-1}) and 20 μl of Tris-acetate buffer of varying pH (1 M). After 90 min incubation at 37 °C, samples were treated as described under "BABQ binding to DNA and polynucleotides". Similar experiments have also been performed with unreduced BABQ.

To study the influence of glutathione and cysteine on the protein binding, 100 μl of the 225 μM [^{14}C]BABQ solution was added to 20 μl Tris-acetate buffer (pH 8.0) (1 M) and 100 μl of a mixture of 1.5 mg · ml^{-1} bovine albumin and 225 μM glutathione (pH 8.0) or 225 μM cysteine (pH 8.0). After 1 h incubation at 37 °C, samples were treated as described under "BABQ binding to DNA and polynucleotides".

To study the pH-dependence of binding of BABQ to glutathione, 100 μl of the 225 μM [^{14}C]BABQ solution was added to a mixture of 20 μl Tris-acetate buffer of varying pH (1 M) and 100 μl glutathione solution (225 μM, pH adjusted to corresponding value). After 2-h incubation at 37 °C, the samples were analyzed by HPLC using 5 mM Tris-acetate buffer (pH 7.6) as eluent. Detection was at 340 nm. After this, the column was washed with methanol to elute BABQ and its decomposition products. The buffer-eluted fractions, containing glutathione-BABQ adducts (see Results and Discussion), were collected, adjusted to a volume of 10 ml and scanned spectrophotometrically. The amount of glutathione adduct present in the sample was assayed using the absorption at 340 nm.

Binding of BABQ to bacterial macromolecules in situ

Overnight cultures of *E. coli* strains (10 ml) were washed twice with 10 ml

of glucose-phosphate buffer (pH 7.0) and incubated in 10 ml of glucose-phosphate buffer (pH 7.0) at 37 °C under shaking. The titer of the *E. coli* suspension was determined by plating. [^{14}C]BABQ stock solution was added to a final concentration of 10 μM. After 90-min incubation, 5-ml samples were taken and put on ice. After washing with ice-cold glucose 5%, the samples were suspended in 5 ml of ice-cold TCA 5%, and put on ice overnight. The suspension was fractionated as described by de Mol et al. [19]. The amount of radioactivity in the RNA, DNA and protein fractions was determined by liquid scintillation counting. The protein content of each fraction was determined by the method of Lowry-Hartree [20]. The phosphor content of each fraction was determined by the method of Lindberg and Ernster [21]. Corrections for small amounts of nucleic acid in the protein fraction and for small amounts of protein in the nucleic acid fractions were made when calculating the amount of radioactivity bound per mg RNA, DNA or protein.

RESULTS AND DISCUSSION

Binding of BABQ to calf thymus DNA and polynucleotides involves a pH dependent reaction: at low pH, alkylation is enhanced (Fig. 2). Electrochemical reduction further promotes binding: after electrochemical reduction, binding is already observable at physiological pH (Fig. 2). It appears that alkylation of DNA and polynucleotides can be achieved by both reduced and unreduced BABQ at low pH, but that reduction greatly enhances the number of adducts.

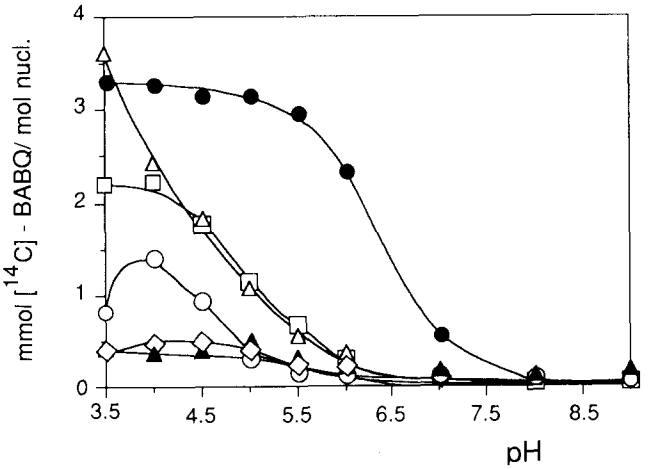

Fig. 2. pH-dependence of binding of electrochemically reduced BABQ to calf thymus DNA and synthetic polynucleotides. BABQ was completely reduced at pH 7.0; samples contained 100 μM reduced [^{14}C]BABQ, 90 μM Tris-acetate buffer and 1.76 mM DNA or polynucleotide, calculated as phosphate and were incubated for 90 min at 37 °C. □, calf thymus DNA; △, poly(dGdC) poly(dGdC); ○, poly(dAdT) · poly(dAdT); ▲, poly(dG) · poly(dC); ●, poly(dA) · poly(dT); ◇, binding of unreduced BABQ to calf thymus DNA.

These results are in agreement with earlier observations [8] where the UV absorbance maximum of alkylated DNA at 343 nm was used to measure the amount of alkylation. We have obtained similar results by studying DNA interstrand cross-linking by BABQ and BABQ derivatives [8]. The increased alkylation and cross-linking of DNA by BABQ at lower pH indicates that protonation of the aziridine ring is an essential step for alkylation; this is in agreement with a high reactivity of protonated aziridines towards nucleophilic species [22]. The reaction mechanism, involving the presence of an aziridinium ion intermediate, is analogous to that of nitrogen mustard compounds [23].

Reduction of BABQ activates the compound by making the quinone moiety electron rich; this facilitates protonation of the aziridine rings and subsequent ring opening and alkylation.

When the pH-binding profiles of polynucleotides are compared with those of DNA (Fig. 2), the same pH-dependence is observed. However, the extent of alkylation varies: binding to poly(dA · dT) is lower than binding to DNA, whereas binding to poly(dG · dC) equals or slightly exceeds binding to DNA (Fig. 2). Binding to poly(dG) · poly(dC) was lower than to the other polynucleotides, whereas binding to poly(dA) · poly(dT) reached its maximum value at a higher pH than the other polynucleotides or calf thymus DNA (Fig. 2).

Nucleotide specificity of alkylating compounds containing an aziridine group or nitrogen mustard function has been studied by several authors. Mitomycin C binds via aziridine ring opening preferentially to the N2 position of guanine in calf thymus DNA and poly(dG · dC) after reductive activation and to the N7 position of guanine after acid activation [24]. Nornitrogen mustard and aziridine ethanol bind preferentially to the N7 position of guanosine and guanine in calf thymus DNA [25]. More specifically, nitrogen mustards bind preferentially to runs of contiguous guanines in pBR322 DNA [26]. These results can be explained by the presence of a negative molecular electrostatic potential near the reaction site [27], which facilitates attack by a positively charged species. Using molecular orbital calculations, the N7 position of guanine was found to be the preferred alkylation site for protonated aziridine, where the O6 atom of guanine is involved in base-pair hydrogen bonding [28]. In view of the similarity between the reactive intermediates of BABQ and nitrogen mustards, the same preference for alkylation on guanine could be expected in the case of BABQ. Our results on binding to polynucleotides do not unambiguously support preferential binding to guanine residues. The increased binding to poly(dG · dC) compared to poly(dA · dT) is in agreement with guanine preference, however the efficient binding to poly(dA) · poly(dT) is not. Possibly in this latter case steric factors could be important, e.g. poly(dA) · poly(dT) is a helix with a distinctively shorter pitch and a narrower minor groove than normal B-form DNA [29].

An attempt was made to identify the nature of the BABQ adduct(s) by enzymatic digestion of DNA to nucleosides after reaction with BABQ. Before digestion, unbound BABQ products were removed by Sephadex filtration and extractions. After digestion, modified nucleosides were separated by

HPLC, first using acetic acid (pH 3.3) as eluent and detection at 343 nm, which is the UV maximum of DNA-BABQ adduct before digestion. Digestion of unreacted calf thymus DNA yielded no products with UV absorbance at 343 nm, therefore peaks with absorbance at 343 nm are ascribed to BABQ adducts. Two principal peaks eluted with absorbance at 343 nm (peak A at 39% methanol and peak B at 53% methanol; Fig. 3). These two peaks were quantitatively obtained from repeated runs (yielding fractions A and B), and lyophilized and again analyzed by HPLC, this time using Tris buffer (pH 7.2) as eluent and detection at 343 and 275 nm. Fraction A appeared to consist of two components that had not been separated at pH 3.3: a compound with UV absorbance around 275 nm (peak C) and a product which showed absorbance

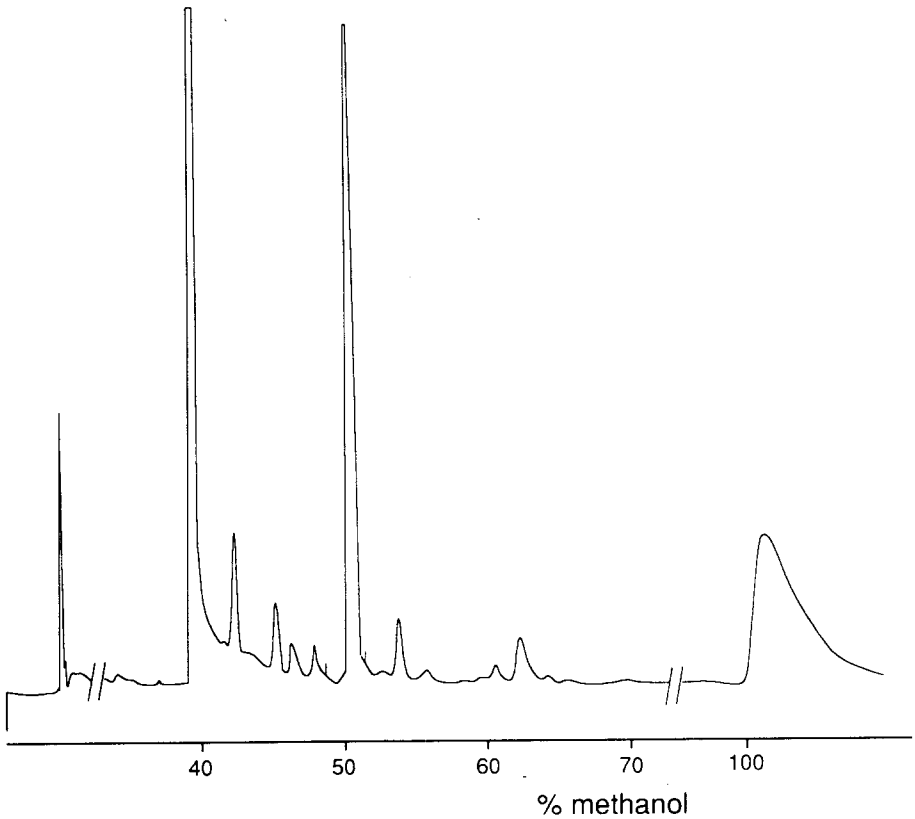

Fig. 3. HPLC chromatogram of digested BABQ-DNA adduct, obtained by electrochemical reduction of a mixture containing 0.5 mg · ml^{-1} calf thymus DNA and 2 mM BABQ in 50 mM phosphate buffer (pH 6.0) and digested with DNAse I, nuclease P1 and alkaline phosphatase, as described in Materials and Methods. Gradient elution was from 100% acetic acid (15 mM; pH 3.3) to 100% methanol; detection was at 343 nm.

at 343 nm (peak D). The retention time and UV spectrum of the compound of peak D were identical to that of 2,5-bis(ethanolamino)-1,4-benzoquinone (Fig. 4, broken line). Fraction B appeared to consist of a peak eluting at 47% methanol (peak E), a peak (F) which had the same retention time as peak D (39% methanol) and a peak (G) at 33% methanol with absorbance at 275 nm. Incubation with acid did not result in further degradation of fraction B. The UV spectrum of peak E is given in Fig. 4 (solid line). In addition to the UV maximum at 343 nm, a maximum at 287 nm is observed at neutral pH and at 274 nm at pH 3.3. The UV maxima indicate that the compound is a BABQ adduct, with extra UV maxima at wavelengths where nucleosides show absorbance. At alkaline pH, the UV maximum shifts towards 320 nm, indicating substitution of (ring opened) aziridine groups by hydroxyl groups, as described by Kusai et al. [30]. During the isolation of peak E, a great loss in UV absorption at 343 nm occurred. Mass spectrometry confirmed the identity of compound D (*m/z* = 192; bis(ethanolamino)-1,4-benzoquinone after loss of OH-groups), but could not reveal the identity of compound E due to its

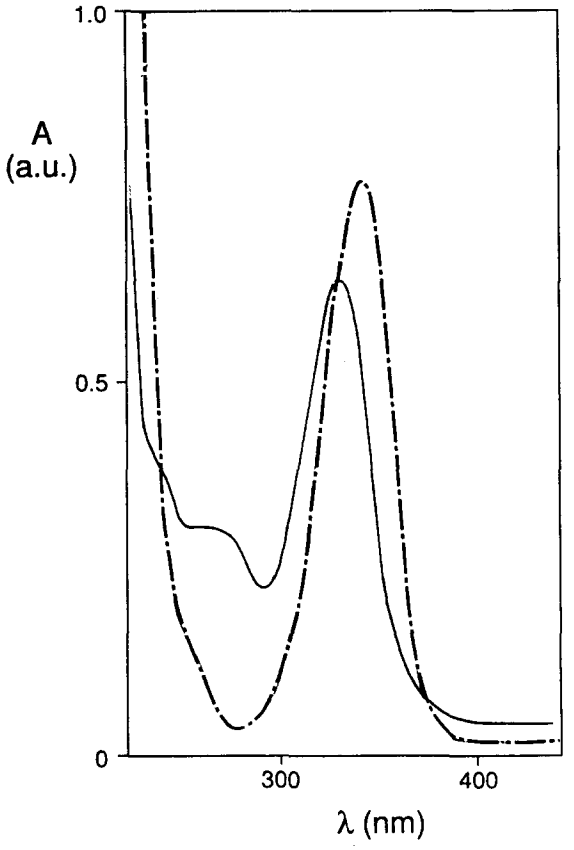

Fig. 4. Broken line: UV spectrum of 2,5-bis(ethanolamino)-1,4-benzoquinone; solid line: UV spectrum of peak E (see text).

instability. Peak G could be a (modified) DNA base, resulting from adduct decomposition. However, the absorption spectrum of compound G was not identical to that of one of the principle DNA bases, nor were its HPLC elution properties. Unfortunately peak G was not obtained in sufficient purity to allow unambiquous interpretation of its mass spectrum. Nevertheless, these results indicate that there is one (unstable) main nucleoside adduct from BABQ alkylation. This nucleoside adduct probably contains BABQ with opened aziridine rings, in view of the similarity of its UV maximum with ring-opened BABQ (Fig. 4).

The observed instability of the adduct is remarkable, as adducts from nitrogen mustard derivatives are readily obtained by enzymatic or acid hydrolysis [25,31].

To study whether BABQ semiquinone or BABQ hydroquinone is involved in the alkylation of DNA, the binding of BABQ to calf thymus DNA was followed at pH 5.0 under several reducing conditions. Electrochemical reduction of BABQ in aqueous medium produces the two-electron reduced species (hydroquinone), whereas the one-electron reduced species (semiquinone) can only directly be produced electrochemically, by reduction in non-aqueous media [32,33]. However, the semiquinone is present in a dismutation-comproportionation equilibrium with hydroquinone and quinone (Eqn. 1), as demonstrated for e.g. diaziquone [34,35]:

$$2Q^{\cdot-} \rightleftharpoons Q + Q^{2-} \qquad (1)$$

Three time-dependent experiments were performed: (a) Incubation of BABQ with DNA under nitrogen, without electrochemical reduction (only oxidized BABQ present). (b) Incubation of BABQ with DNA under nitrogen, while BABQ is reduced during incubation with DNA, as indicated by the decreasing cathode current (Fig. 5). In this case DNA is exposed to BABQ semiquinone formed in a dismutation equilibrium (Eqn. 1) and to BABQ hydroquinone. (c) Incubation under nitrogen, while DNA is added after reduction of BABQ and BABQ is kept reduced electrochemically (low cathode current, Fig. 5). In this experiment, DNA is exposed to only BABQ hydroquinone. The results are represented in Fig. 5. When BABQ is incubated with DNA without electrochemical reduction (Expt. a), binding of BABQ to calf thymus DNA is low. Electrochemically reduced BABQ, incubated with DNA at pH 5.0 under nitrogen (Expt. c), binds to DNA somewhat more than non-reduced BABQ. At pH 5.0 BABQ hydroquinone is less stable than at pH 7.0, therefore reduction occurred at pH 7.0. At aeration after incubation at pH 5.0 with DNA, an increase in binding is observed (results not shown). This indicates that part of the alkylating aziridinyl groups remained intact during incubation at pH 5.0 and that the low binding in Expt. c is not caused by loss of the aziridinyl group by ring opening. During electrochemical reduction of BABQ in the presence of DNA (Expt. b), binding to DNA takes place most efficiently. Under these conditions DNA is able to react with BABQ semiquinone; we therefore conclude that BABQ semiquinone is the most reactive

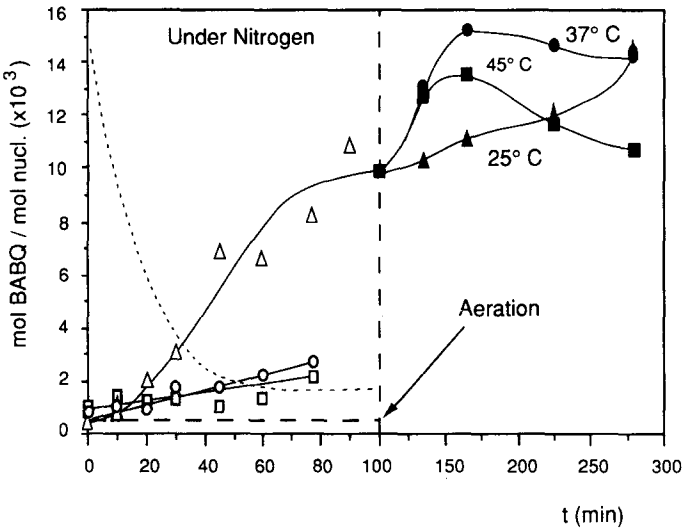

Fig. 5. Time-dependence of binding of BABQ to calf thymus DNA at pH 5.0 under nitrogen. Samples contained 225 μM [^{14}C]BABQ, 0.6 mg/ml calf thymus DNA and 5 mM Tris-acetate buffer (pH 5.0). □, binding of unreduced BABQ (Expt. a); △, binding during electrochemical reduction of BABQ (Expt. b); ○, binding of electrochemically reduced BABQ (Expt. c); - - -, cathode current of Expt. b; — —, cathode current of Expt. c. At t = 105 min, Expt. b was continued under air, without further reduction, after pH adjustment to 7.0. Samples were incubated at 25°C (▲); 37°C (●) and 45°C (■).

species in the alkylation reaction with DNA. Electrochemical reduction of BABQ as used to study DNA binding (e.g. see Fig. 2) yields two electron reduced BABQ (hydroquinone), from which in the presence of oxygen the alkylating semiquinone is formed by reoxidation and dismutation (Eqn. 1). Analogous to our results is the observation of Nguyen et al. [34], that a diaziquone radical generating mixture has a higher growth inhibiting activity on P388 cells than diaziquone.

Alkylation by the semiquinone form of quinoid antitumour drugs like adriamycin [36], mitomycin C [37] or more simple quinones like menadione [38] has been described in the literature. With simple quinones, direct reaction of the quinone moiety is involved [39]. However, in the case of BABQ the aziridinyl group is the alkylating function: this is demonstrated by the necessity of two aziridine groups for the formation of DNA interstrand crosslinks [8] and the pH dependence of DNA alkylation as described in this paper.

The pK_a value of the aziridine ring is expected to increase upon reduction, as reduction increases the electron density in the BABQ molecule. Two-electron reduction will obviously increase the overall electron density to a larger extent than one-electron reduction. However, this does not lead to a higher

alkylating activity of two-electron reduced BABQ compared to BABQ semiquinone. Possibly the pK_a value of the aziridine ring does not simply correlate with total molecular electron density. Conformational changes might be related with the reduction process, e.g. from ab initio calculations at the STO-3G level it appears that the position of the aziridine ring with respect to the benzoquinone ring changes upon reduction due to the formation of hydrogen bonds (E.E. Moret, to be published).

The binding process in Expt. b was further followed, after stopping the reduction process and aeration, at different temperatures. During aeration, DNA alkylation continues and is dependent upon the temperature. At 25°C, binding continues for at least 180 min, but eventually decreases (Fig. 5). At 37°C and 42°C, DNA alkylation continues during approx. 45 min and then declines (Fig. 5). The continuation of DNA alkylation after termination of electrochemical reduction and exposing the reaction mixture to air can be explained by the formation of semiquinone radicals during autoxidation of BABQ hydroquinone. This process continues as long as BABQ hydroquinone reoxidizes. However, the decrease of covalent binding indicates that the DNA adduct is not stable; decomposition of the adduct is faster at elevated temperatures. These results are in agreement with the difficulties encountered with the isolation of nucleoside adducts as described above.

In contrast with the observed pH dependence for BABQ binding to DNA and polynucleotides (Fig. 2), binding of BABQ to the protein BSA increases at higher pH values (Fig. 6). Binding to BSA is not increased by electrochem-

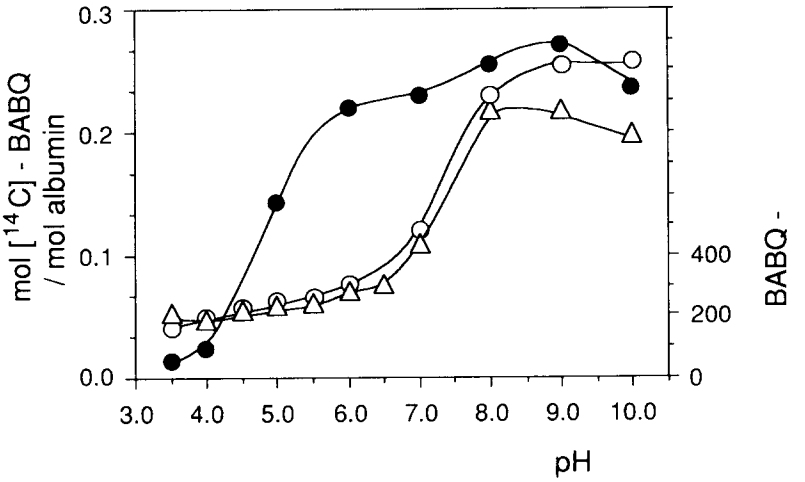

Fig. 6. pH-dependence of binding by BABQ to bovine serum albumin. Samples contained 100 μM reduced or non reduced [^{14}C]BABQ (BABQ was completely reduced at pH 7.0), 90 μM Tris-acetate buffer and 0.68 mg/ml albumin and were incubated for 90 min at 37°C (O, BABQ; △, electrochemically reduced BABQ). ●, pH-dependence of BABQ adduct formation with glutathione. These samples contained 100 μM [^{14}C]BABQ, 100 μM glutathione and 100 mM Tris-acetate buffer and were incubated for 120 min at 37°C. HPLC fractions eluted with tris buffer were collected, made up to 10 ml volume, and measured for UV absorption at 340 nm.

ical reduction of BABQ when the reaction pH is under 8.0 and even decreases at higher pH-values. Addition of glutathione and cysteine inhibits the binding of BABQ to BSA at pH 8.0 (Table I). Binding of BABQ to BSA evidently involves a reaction mechanism that is essentially different from that of binding to DNA. Attack by the (protonated) aziridinyl group can not be involved in binding to BSA, as neither protonation nor reduction lead to increased binding. The partial inhibition by glutathione and cysteine of BABQ binding to BSA indicate that in the nucleophilic addition with BABQ, —SH groups might be involved. The observed increase in binding of BABQ to BSA around pH 7.0—7.5 may also reflect a change in the conformation of BSA as described for human serum albumin, the so-called neutral-to-base or N-B transition between pH 6 and 9, which affects drug binding [40].

HPLC analysis of a mixture of 100 μM BABQ, 100 μM glutathione and 100 μM Tris (pH 8.0), incubated at 37 °C during 40 min (Fig. 7), revealed that apart from glutathione, several products with absorbance at 340 nm eluted with 5 mM Tris buffer (pH 7.4), which had UV maxima near 345 nm. These peaks are not BABQ or BABQ decomposition products resulting from acid or alkali hydrolysis, as these are eluted only with methanol-buffer mixtures. Peak A shows UV maxima at 340 and 252 nm; peak B shows UV maxima at 350 and 258 nm. The UV maximum at higher wavelengths indicates the presence of a BABQ product and is close to the UV maximum of aziridine ring opened BABQ bis(ethanolamino)benzoquinone, at 343 nm [30]. In view of these spectral properties and the retention times, these peaks are ascribed to glutathione-BABQ adducts. The total amount of glutathione adducts was assessed by measuring the UV absorbance at 340 nm of the Tris eluate, made up to a fixed volume. As in the case of BABQ binding to BSA, the amount of water-soluble BABQ adducts increases with increasing pH (Fig. 6). This increase in binding at higher pH-values is in accordance with deprotonation of the SH-group, which facilitates attack by a nucleophile [41]. The pH-value where UV absorbance at 340 nm is half-maximal, however, is substantially lower than the pK_a value of 8.8 for glutathione [42]. Binding of SH-groups to benzoquinones and naphthoquinones by nucleophilic attack,

TABLE I

EFFECTS OF GLUTATHIONE AND CYSTEINE ON THE BINDING OF BABQ TO BSA

Samples containing 100 μM BABQ, 0.6 mg/ml BSA, 100 mM Tris-acetate buffer (pH 8.0) and 100 μM glutathione or cysteine, as indicated, were incubated for 1 h at 37 °C.

Component added to the incubation mixture	Binding of BABQ to BSA (mmol BABQ/mol albumin)
Control	283 ± 12
100 μM glutathione	134 ± 6
100 μM cysteine	144 ± 12

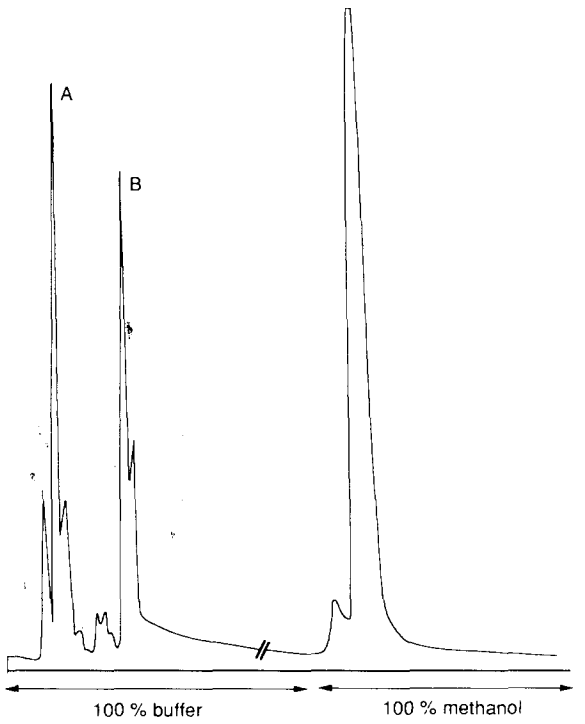

Fig. 7. HPLC chromatogram of a reaction mixture of 100 μM BABQ, 100 μM glutathione and 100 mM Tris-acetate buffer pH 8.0, incubated at 37 °C for 40 min. Eluent was 100% tris buffer (5 mM; pH 7.4)/100% methanol. Detection was at 340 nm.

leading to formation of a hydroquinone adduct, has been described e.g. by Rossi et al. [43] and Ross et al. [44].

The covalent binding of BABQ to proteins, DNA and RNA in intact cells was studied in *E. coli* bacteria. We used the same *E. coli* strains previously in a differential DNA repair test [12]: *E. coli* 343/753 is deficient in DNA repair and *E. coli* 343/765 has wild type DNA repair. In *E. coli*, BABQ is reductively activated by bacterial reductases. Table II shows the results of binding by BABQ to cellular fractions of both *E. coli* strains. Extensive protein binding was observed in both strains. BABQ is readily taken up by *E. coli*, or associates rapidly to the cells, as an increase in total radioactivity in *E. coli* could only be observed during the first 10 min of incubation. The DNA repair deficient strain shows a slightly higher binding to all cellular fractions. The DNA-repair deficient strain is killed more easily [12] due to DNA alkylation and the absence of certain DNA repair enzymes. However, a higher amount of alkylated DNA was not demonstrated in this strain.

In conclusion, one-electron reduction of BABQ to the semiquinone appears to be the activating step for DNA alkylation, while two-electron reduction leads to a relatively unreactive species, that can be conjugated cellularly and

TABLE II

BINDING OF BABQ TO CELLULAR FRACTIONS OF *E. COLI* K12-343/753 (DNA REPAIR DEFICIENT) AND 343/765 (WILD TYPE DNA REPAIR)

Cell suspensions containing 8×10^8 cells/ml were incubated at 37 °C in glucose-phosphate buffer (pH 7.0) in the presence of 10 μM BABQ under shaking for 30 min.

Cellular fraction	Binding to *E. coli* K12-343/765	Binding to *E. coli* K12-343/753
RNA	1.32 mmol/mol nucl.	1.78 mmol/mol nucl.
DNA	1.22 mmol/mol nucl.	1.99 mmol/mol nucl.
Protein	40.0 mmol/mol	58.4 mmol/mol

excreted. Binding of BABQ to albumin proceeds by a reaction mechanism that, in contrast to DNA alkylation, does not involve aziridine ring protonation and opening. In *E. coli*, appreciable protein binding next to DNA binding is demonstrated. The difference in reaction chemistry between alkylation of DNA and of protein is interesting from the viewpoint of drug design: the specificity of BABQ for DNA alkylation relative to protein alkylation increases at lower pH values. This offers perspectives for the development of more specifically acting BABQ derivatives. When certain tumour cells are more acidic and hypoxic than normal cells, this type of drug might have more specific activity with respect to DNA alkylation in these cells.

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REFERENCES

1 G. Domagk, S. Petersen and G. Gauss, Experimental chemotherapy of tumors, Z. Krebsforsch., 59 (1954) 617–622.
2 G. Domagk, Histological changes in experimental and human tumors after administration of cytostatics agents, Deut. Med. Wochenschr., 81 (1956) 801–806.
3 J.S. Driscoll, L. Dudeck, G. Congleton and R.I. Geran, Potential CNS antitumor agents IV: aziridinylbenzoquinones III, J. Pharm. Sci., 68 (1979) 185–188.
4 R.T. Eagan, R.P. Dinapoli, T.L. Cascino, B. Scheithauer and B.P. O'Neill, Comprehensive Phase II evaluation of aziridinylbenzoquinone (AZQ, diaziquone) in recurrent human primary brain tumors, J. Neurooncol., 5 (1978) 309–314.
5 M.C. Chamberlain, M.D. Padros, P. Silver and V.A. Levin. A phase I/II study of 24 h intravenous AZQ in recurrent primary brain tumors, J. Neurooncol., 6 (1988) 319–323.
6 A.J. Lin, L.A. Cosby, X. Shanky and A.C. Sartorelli, Potential bioreductive alkylating agents. 1. Benzoquinone derivatives, J. Med. Chem., 12 (1972) 1246–1252.
7 G. Powis, Metabolism and reactions of quinoid anticancer agents, Pharmacol. Ther., 35 (1987) 57–162.
8 K.J. Lusthof, N.J. de Mol, L.H.M. Janssen, W. Verboom and D.N. Reinhoudt, DNA alkyla-

tion and formation of DNA interstrand cross-links by potential antitumor 2,5-bis(1-aziridinyl)-1,4-benzoquinones, Chem.-Biol. Interact., 70 (1989) 249–262.
9 K.A. Kennedy, B.A. Teicher, S. Rockwell and A.C. Sartorelli, The hypoxic tumor cell: a target for selective cancer chemotherapy (Commentary), Biochem. Pharmacol., 29 (1980) 1–8.
10 W.A. Denny and W.R. Wilson, Considerations for the design of nitrophenyl mustards as agents with selective toxicity for hypoxic tumor cells, J. Med. Chem., 29 (1986) 879–887.
11 J.L. Wike-Hooley, J. Halveman and H.S. Reinhold, The relevance of tumor pH to the treatment of malignant disease. Radiother. Oncol., 2 (1984) 343–366.
12 K.L. Lusthof, N.J. de Mol, L.H.M. Janssen, W. Verboom and D.N. Reinhoudt, Potential antitumor 2,5-bis(1-aziridinyl)-1,4-benzoquinones: Relationship between cytotoxic against DNA repair deficient *E. coli*, inactivation of bacteriophage M13mp19 and in vitro and in vivo antitumour activity, Anti-Cancer Drug Design, 3 (1988) 147–155.
13 British patent no. 762723 Farb. Fab. Bayer A.G., (1-Aziridinyl) substituted quinones and processes for their preparation, Chem. Abstr., 51 (1956) 12153h.
14 R.D. Wells, J.E. Larsson and R.C. Grant, Physicochemical studies on polydeoxyribonucleotides containing defined repeating nucleotide sequences, J. Mol. Biol., 54 (1970) 465–497.
15 G.R. Mohn, P.R.M. Kerklaan, A.A. van Zeeland, J. Ellenberger, R.A. Baan, P.H.M. Lohman and F.W. Pons, Methodologies for the determination of various genetic effect in permeable strains of *E. coli* K-12 differing in DNA repair capacity, Mutat. Res., 125 (1984) 153–157.
16 V. Massey and P. Hemmerich, A photochemical procedure for reduction of oxidation-reduction proteins employing deazariboflavin as catalyst, J. Biol. Chem., 252 (1977) 5612–5614.
17 T. Maniatis, E.F. Fritsch and J. Sambrook, In: Molecular Cloning. A Laboratory Manual, CSH Laboratory, Cold Spring Harbor, New York, 1982, pp. 466–467.
18 G.W. Gehrke, Quantitative reversed-phase high performance liquid chromatography of major and modified nucleosides in DNA, J. Chromatogr., 301 (1984) 309–314.
19 N.J. de Mol and R.W. Busker, Irreversible binding of the chlorpromazine radical cation and of photoactivited chlorpromazine to biological macromolecules, Chem.-Biol. Interact., 52 (1984) 79–92.
20 E.F. Hartree, A Modification of the lowry method that gives a linear photometric response, Anal. Biochem., 48 (1972) 422–427.
21 O. Lindberg and L. Ernster, Methods of Biochemical Analysis, 3 (1956) 1–22.
22 O. Kikuchi, A.J. Hopfinger and G. Klopman, Chemical reactivity of protonated aziridine with nucleophilic centers of DNA bases, Biopolymers, 19 (1987) 325–340.
23 C.E. Williamson and B. Witten, Reaction mechanism of some aromatic nitrogen mustards, Cancer Res., 27 (1967) 33–38.
24 M. Tomasz, R. Lipman, M.S. Lee, G.L. Verdine and K. Nakanishi, Reaction of acid-activated mitomycin C with calf thymus DNA and model guanines: elucidation of the base-catalyzed degradation of N7-alkylguanine nucleosides, Biochemistry, 26 (1987) 2010–2027.
25 K. Hemminki, DNA binding products of nitrogen mustard, a metabolite of cyclophosphamide, Chem.-Biol. Interact., 61 (1987) 75–88.
26 W.B. Mattes, J.A. Hartley and K.W. Kohn, DNA specificity of guanine-N7 alkylation by nitrogen mustards, Nucl. Acid. Res., 14 (1986) 2971–2987.
27 K.W. Kohn, J.A. Hartley and W.B. Mattes, Mechanisms of DNA sequence selective alkylation of guanine-N7 positions by nitrogen mustards. Nucl. Acids Res., 15 (1987) 10531–10549.
28 O. Kikuchi and A.J. Hopfinger, Chemical reactivity of protonated aziridine with nucleophilic centers of DNA bases, Biopolymers, 19 (1980) 325–340.
29 D.G. Alexeev, A.A. Lipanov and I.Ya Skuratovskii, Poly(dA) · poly (dT) is a B-type double helix with a distinctively narrow minor groove, Nature, 325 (1987) 821–823.
30 A. Kusai, S. Tanaka and S. Ueda, The stability of carboquone in aqueous solution. I. Kinetics and mechanisms of degradation of 2,5-diethylenimino-1,4-benzoquinone in aqueous solution, Chem. Pharm. Bull., 29 (1981) 3671–3679.
31 J.R. Mehta and D.B. Ludlum, Trapping of DNA-reactive metabolites of therapeutic or car-

cinogenic agents by carbon-14 labeled synthetic polynucleotides, Cancer Res., 42 (1982) 2996–2999.

32 M.M. Mossoba, M. Alizadeh and P.L. Gutierrez, Mechanism for the reductive activation of diaziquone, J. Pharm. Sci., 74 (1985) 1249–1254.

33 R.C. Prince, P. Lloyd-Williams, J.M. Bruce and P.L. Dutton. Voltammetric measurements of quinones, Methods. Enzymol., 125 (1986) 109–119.

34 B. Nguyen, S. Biswal and P.L. Gutierrez, The influence of diaziquinone free radicals on the in vitro activity of diaziquone, Xenobiotica, 18 (1988) 593–602.

35 P.L. Gutierrez, M. Balachandran, M.S. Nardino and P.S. Callery, The chemical reduction of diaziquone: products and free radical intermediates, Chem.-Biol. Interact., 64 (1987) 23–37.

36 B.K. Sinha, Binding specificity of chemically and enzymatically activated anthracycline anticancer agents to nucleic acids, Chem.-Biol. Interact., 30 (1980) 67–77.

37 A.S. Sartorelli, The role of mitomycin antibiotics in the chemotherapy of solid tumors, Biochem. Pharmacol., 35 (1986) 67–69.

38 H. Morrison, J. Jernström, M. Nordenskjöld, H. Thor and S. Orrenius, Induction of DNA damage by menadione (2-methyl-1,4-naphthoquinone) in primary cultures of rat hepatocytes, Biochem. Pharmacol., 33 (1984) 1763–1769.

39 D. Ross, H. Thor, M.D. Threadgill, M.S. Sandy, M.T. Smith, P. Moldeus and S. Orrenius, The role of oxidative processes in the cytotoxicity of substituted 1,4-naphthoquinones in isolated hepatocytes, Arch. Biochem. Biophys., 248 (1986) 460–466.

40 J. Wilting. W.F. van der Giessen, L.H.M. Janssen, M.M. Weideman, M. Otagiri and J.H. Perrin, The efiect of albumin conformation on the binding of warfarin to human serum albumin, J. Biol. Chem., 255 (1980) 3032–3037.

41 I. Wilson, P. Wardman, T. Lin and A.C. Sartorelli, Reactivity of thiols towards derivatives of 2- and 6-methyl-1,4-naphthoquinone bioreductive alkylating agents, Chem.-Biol. Interact., 61 (1987) 229–240.

42 D.D Perrin, In: Dissociation Constants of Organic Bases in Aqueous Solution, suppl. 1972, no7631, Butterworths, London, 1972.

43 L. Rossi, G.A. Moore, S. Orrenius and P.J. O'Brien, Quinone toxicity in hepatocytes without oxidative stress, Arch. Biochem. Biophys., 251 (1986) 25–35.

44 D. Ross, H. Thor, S. Orrenius and P. Moldeus, Interaction of menadione (2-methyl-1,4-naphthoquinone) with glutathione, Chem.-Biol. Interact., 55 (1985) 177–184.